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The existence of prostate stem cell capable of giving rise to all the epithelial lineages present in the adult prostate is very controversial. Understanding the stages of cell differentiation in normal prostate epithelium is essential for the identification of the cell type(s) involved in prostate carcinogenesis. The p53-homologue p63 is selectively expressed in the basal cell compartment of a variety of epithelial tissues and p63 deficient mice show severe defects in the development of epithelial organs, including agenesis of the prostate. These findings suggest that p63 is required to maintain a prostate stem cell population. In order to test this hypothesis we will first study p63 expression in the various stages of prostate development in wild type mice by both immunohistochemistry and in situ hybridization (Specific Aim 1). We will also construct chimeric mice by injecting p63+/+ β-galactosidase positive ES cells into p63-/- blastocysts (Specific Aim 2) and then analyze the relative contribution of p63+/+ and p63-/- cells to the prostatic epithelium. In the event in which both basal and secretory cells require p63 for development, the results will indicate that both compartments originate from a common p63-positive stem cell.

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INTRODUCTION

One substantial limitation in understanding the molecular events that lead to prostate cancer is that the cell type undergoing neoplastic transformation in the prostate is unknown. Moreover, although three major cell types have been identified within the prostate epithelium, the hierarchical relation between them remains obscure and the existence of prostate stem cells is uncertain (1-6). It appears clear that unraveling the epithelial hierarchy in the normal prostate epithelium has important implications in identifying the cell of origin of prostate carcinoma and its pathogenetic mechanisms. The aim of this proposal is to identify prostate stem cells.

The p53 homologue p63 is selectively expressed in the basal cell compartment of the several epithelia, including the prostate. p63 knock-out mice show severe defects in the development of epithelial organs, including the agenesis of all squamous epithelia, breasts, salivary glands and lachrymal glands (7,8). We have recently demonstrated that do not develop the prostate (9). These findings imply that during embryogenesis p63 is required to maintain an epithelial cell population that plays a crucial role in prostate morphogenesis. Two main hypotheses can explain the defect in prostate development in p63-/- mice: 1) p63 is essential for maintaining a prostate epithelial stem cell population that generates both basal and secretory cells 2) p63 is essential for maintaining prostate basal cells which do not represent prostate stem cells but are essential for prostate development. In order to test these hypotheses we are constructing chimeric mice by injecting p63+/+ β-galactosidase positive ES cells into p63-/- blastocysts. If, as expected, p63+/+ ES cells abrogate the defect in prostate development, we will analyze the relative contribution of p63+/+ and p63-/- cells to the prostatic epithelium in rescued chimeric mice. This proposal represents a unique approach to resolve the long-standing controversy on the role of prostate basal cells as stem cells. This chimeric model, if successful, may be applied as an innovative approach to study prostate development and neoplastic transformation in vivo. By utilizing genetically altered β-galactosidase positive ES cells (e.g. Rb-/- or p53-/-) it will be possible to generate mice that carry specific genetic alterations targeted to prostate epithelial cells and investigate their role in tumorogenesis and tumor progression.

Body

Research accomplishments based on the approved Statement of Work

Aim 1. To assess the distribution of p63 positive cells in the developing prostate.
The goal of this specific aim is to confirm that the absence of p63 causes arrest in prostate morphogenesis at the stage of budding from the urogenital sinus by comparing prostate development in wild type and p63-/- mice with a detailed morphologic analysis. In addition, by utilizing immunohistochemistry and in situ hybridization, I plan to determine if all cells in the early prostatic buds of wild type mice are p63 positive.
In year 2002, we performed immunohistochemistry for p63 in the urogenital sinus of 10 wild-type male embryos at 18dpc. Our results demonstrate that during this early stage of prostate development, all the cells in the buds are p63 positive. The levels of expression are higher in the cells localized at the periphery of the buds (i.e. the cells in contact with the extracellular matrix).
In situ hybridization experiments are still under optimization. Skin and prostate tissue sections from wild type mice are utilized as positive controls.

Aim 2: To determine which cell compartment(s) require(s) p63 expression for normal prostate development in the mouse.

In order to assess whether or not the p63 positive epithelial cells in the developing prostate represent prostate stem cells that are able to differentiate into both basal and secretory cells, I am utilizing a chimeric mouse system. The aim of this project is to abrogate the defect in prostate development associated with p63 deficiency by injecting p63+/+ ROSA26 ES cells into p63-/- blastocysts and then analyze the relative contribution of p63+/+ and p63-/- cells to the prostatic epithelium of rescued chimeras. It is clear that the success of this animal model is based on two main assumptions: 1) p63 function is cell autonomous 2) limb, skin and prostate defects in the p63-/- mice can be rescued. In the first phase of this project (Y2001) we were able to generate the first chimeric mice and optimize the methodology utilized in the analysis of the chimeras. Our preliminary results suggested a cell-autonomous p63 function, however, our ability to rescue the defects in p63-/- mice had not been yet demonstrated.
In year 2002, we performed two additional experiments. The analysis of the chimeras generated in the first experiment has been completed and the results are described below. We are still analyzing the chimeric mice generated in the second experiment.

Generation of chimeric mice

Experimental design: Fifteen to twenty p63+/- female mice were superovulated and mated to p63+/- male mice. ~140 blastocysts were isolated from 12-15 plugged females. 3.5 dpc blastocysts were injected with ROSA26 ES cells and transferred to 10 foster mothers.

This experiment was designed to analyze an extensive number of chimeric embryos by sacrificing the foster mothers at 18.5dcp. This was done in order to unequivocally identify the phenotype of the chimeras derived from p63-/- blastocysts, which are expected to represent 25% of the embryos. The recovery of the mice before birth allowed us to analyze the entire litter and prevent the mothers from killing un-rescued or partially rescued p63-/- newborns. We decided to sacrifice the embryos at 18.5dpc because prostate buds can be already identified at this stage of development. 56 embryos were generated and fully analyzed in this experiment.

Analysis of 56 chimeric mice at 18.5dpc

Identification of rescued p63-/- blastocysts

Firstly, chimeric mice were analyzed by X-gal histochemistry in order to assess the degree of chimerism in the epidermis, which requires p63 for normal development. As a second step, the level of chimerism was assessed in organs that do not require p63 for normal development, namely the intestine. In all X-gal histochemistry experiments tissue sections from Rosa26 and wild-type mice were utilized as positive and negative controls, respectively. Since our preliminary results (see Y2001 report) suggested that p63 function is cell-autonomous, we hypothesized that in p63-/- rescued chimeras the epidermis would be exclusively composed by ROSA26 cells, while intestine would demonstrate varying degrees of chimerism. We also predicted that in chimeric mice derived from either p63+/+ or +/- blastocysts the contribution of ROSA26 cells would be similar in the epidermis and the intestine. The two predicted scenarios are illustrated in Fig 1.

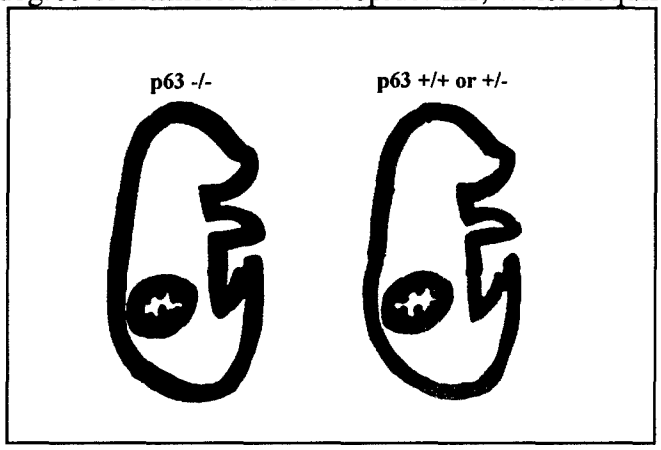

Fig.1. *Expected pattern of staining of epidermal and intestinal epithelial cells in chimeras derived from p63-/- (left) or p63+/+ and p63+/- blastocysts*

X-gal staining in the 56 embryos confirmed our predictions. The results are summarized in Table 1.

Table 1. *X-gal histochemistry was performed on frozen tissue sections of chimeric mice at 18.5dpc. The percentage of epithelial cells positive for beta-gal activity in the epidermis and intestine is reported for each sample.*

N. of Mice	Phenotype	Epidermis	Intestine	Genotype of original blastocyst
4	p63-/-	0	0	p63-/-
8	WT/minor defects	100%	30-70%	p63-/-
42	WT	0-90%	0-90%	p63+/+ or p63+/-
2	WT	100%	100%	Not informative

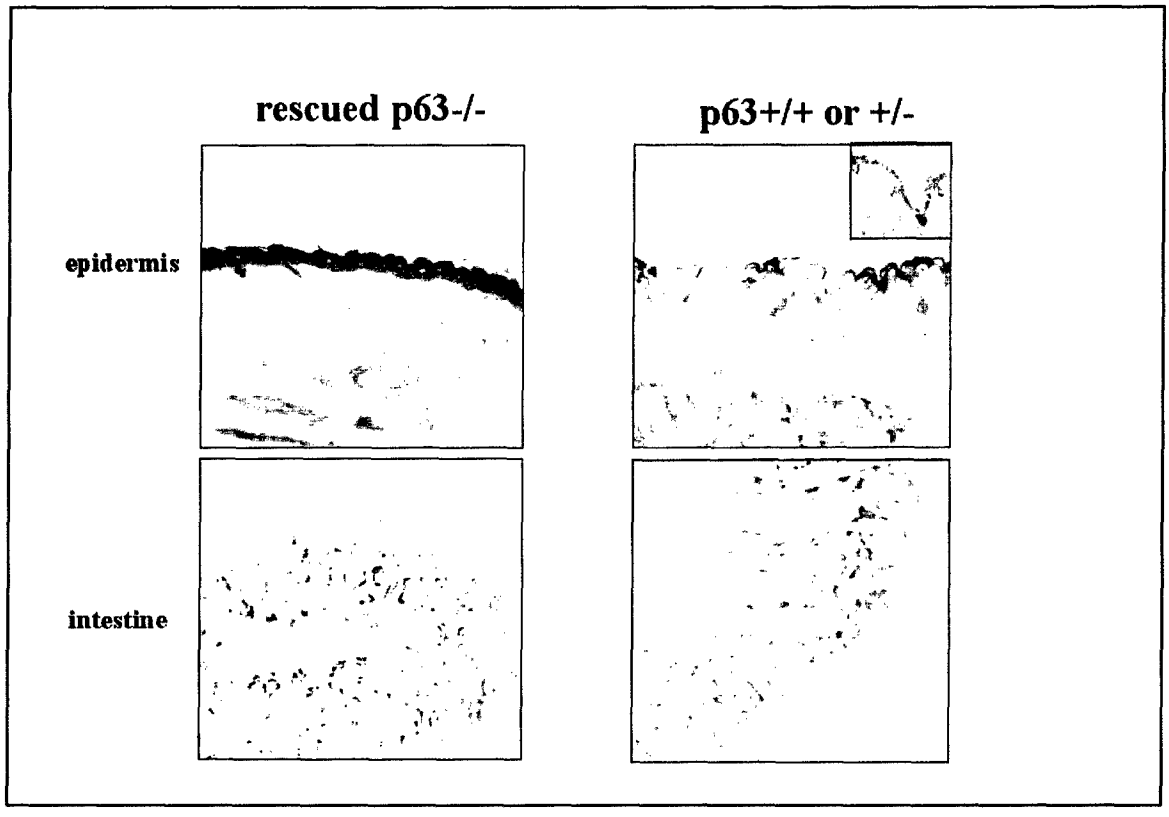

Fig. 2 *Forty-two chimeras presented similar contribution of beta-gal positive cells to the epidermis and the intestine (right panels). In a subset of 8 chimeras the epidermis was exclusively populated by beta-gal positive cells, while a significantly lower level of chimerism was observed in the intestine epithelium (left panels)*

The majority of chimeras (42/56) showed similar levels of chimerism in both the epidermis and intestine epithelium (Fig.2, right panels). However, we also identified a subset of animals (8/56) whose epidermis was exclusively populated by ROSA26 cells while the intestine epithelium (and other p63-unrelated organs) was composed by a significantly lower percentage of ROSA26 cells (Fig.2, left panels). In addition, 4 chimeras showed a typical p63-/- phenotype and, as expected, X-gal staining showed no contribution of ROSA26 cells to these animals. Finally, two embryos consisted exclusively of beta-gal positive (ROSA26) cells and were, therefore, not informative regarding the genotype of the original blastocyst.

Overall, these results suggest that the 42 chimeras previously described originated from either p63+/+ or +/- blastocysts, while the 8 chimeras with an entirely beta-gal positive epidermis represented rescued p63-/- mice. This interpretation is confirmed by two observations.

1) When immunohistochemical staining for p63 was performed in the skin of the 42 chimeras, the beta-gal negative cells (derived from the original blastocyst) consistently expressed p63 protein indicating that p63-/- cells do not contribute to normally developed epidermis and that none of these mice originated from p63-/- blastocysts. This result, besides confirming that p63 function is cell autonomous, indicates that p63-/- blastocysts gave origin to the remaining chimeras. Four animals clearly presented a p63-deficient

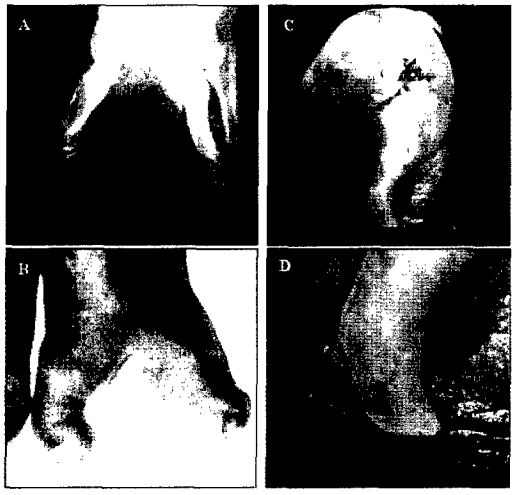

Fig. 3. *Ectrodactyly in a patient affected by EEC syndrome (panels A and B) and limb abnormality in a partially rescued p63-/-embryo (panels C and D).*

phenotype but a larger number of p63-/- blastocysts is expected (~25% of 56 = 14). This is a further indication that the subset of eight chimeras represents the rescued p63-/- mice.

2) More importantly, two of the eight putative rescues presented mild defects in limb development. Specifically, both upper and lower limbs were only partially developed in these mice. Very interestingly, one of the two showed abnormalities (Fig.3, panels C & D) that closely resemble ectrodactyly observed in patients affected by ECC and ECC-related syndrome (Fig.3, panels A & B) These spectrum of syndromes have been recently shown to be caused by mutations in the p63 gene. This result clearly demonstrates that the two malformed chimeric embryos represent partially rescued p63-/-mice and indicates that the additional six chimeras presenting the same pattern of distribution of ROSA26 cells also derive from p63-/- blastocysts. A further unequivocal evidence that these mice represent p63-/- rescues is provided by the analysis of the genitourinary tract of theses embryos (see below).

Analysis of the urogenital sinus and prostate buds

The contribution of beta-gal positive versus beta-gal negative cells was studied in the urogenital sinus epithelium of the 8 rescued p63-/- chimeras. In addition, numerous transversal sections of the urogenital sinus of these mice were stained with H&E and immunostained for p63 for the detection of prostate buds. Whenever the buds were identified, X-gal histochemistry was performed in serial sections to determine the contribution of beta-gal positive cells to these structures. Sections from the UGS of ROSA26 18.5dpc embryos were utilized as positive controls for both X-gal staining and p63 immunohistochemistry.

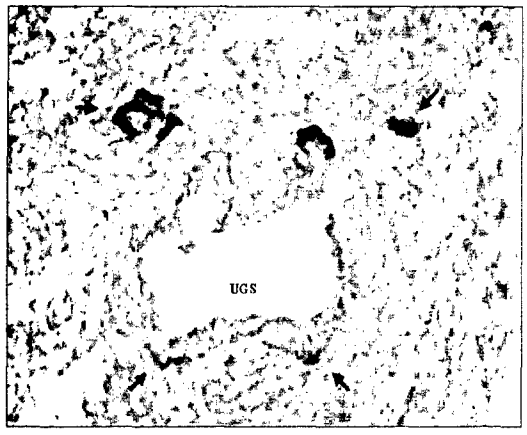

Fig. 4. *Transversal section of the UGS of a p63-/- rescued chimera stained with X-ga.l.*

Our results show that at least one prostate bud could be detected in each of the rescued p63-/- chimeras. By p63 immunohistochemistry, the UGS epithelium of 6/8 rescued chimeras presented focal absence of p63 positive basal cells (Fig.5, upper right panel). This observation implies that the rescue of the UGS

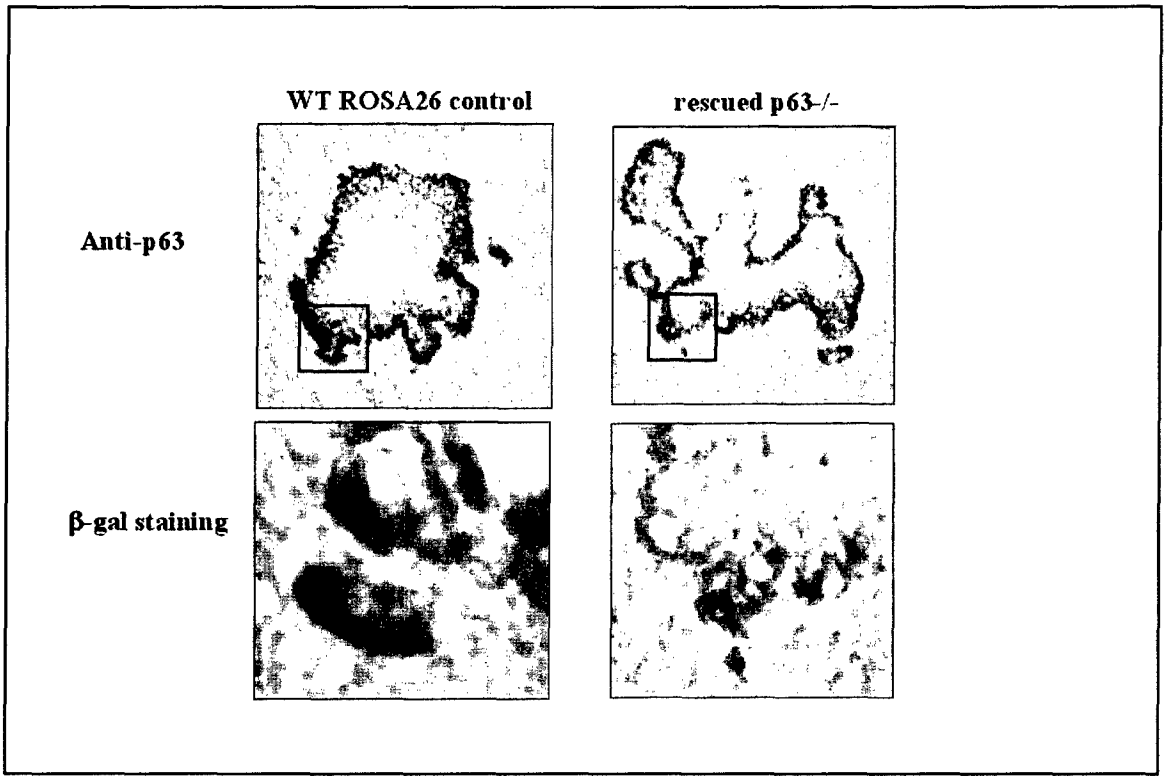

Fig. 5 *Transversal sections of the UGS of a ROSA26 mouse (left panels) and a p63-/- rescued chimera (right panels) were either immunostained for p63 or stained with X-gal*

epithelium is only partial and unequivocally demonstrates that these chimeras derive form p63-/- blastocysts. By X-gal histochemistry, the UGS epithelium of the rescued chimeras consisted of both beta-gal positive and beta-gal negative cells. Interestingly, the prostate buds were exclusively populated by beta-gal positive cells (Fig.4 and Fig.5, lower right panel). As expected, all the epithelial cells in the UGS and prostate buds of a ROSA26 mouse control showed homogeneous beta-gal activity (Fig.5, lower left panel). Overall, these results demonstrate that p63 is required for the development of all the prostate epithelial cells at this early stage of development. However, since all the cells that populate the prostate buds at this stage have a basal cell phenotype and do express p63 (see Aim 1), no information regarding secretory cells can be obtained by analyzing the rescued mice at this embryonic stage. Two different scenarios can be envisioned: 1) the secretory cells develop from p63 +/+, beta-gal positive basal cells present in the buds. In this case the whole prostate epithelium of the post-natal prostate will be constituted by beta-gal positive cells. 2) Alternatively, it is possible that secretory cells do not derive from the basal cells but originate from p63 negative cells present within the UGS epithelium that, further on, colonize the prostate. In this scenario, secretory cells in the fully developed prostate can be p63-/- and, therefore, beta-gal negative. In order to verify which one of these two hypotheses is correct, we have performed a second experiment in which the chimeric mice will be analyzed at 7 weeks of age, when the prostate epithelium is fully developed. The analysis is currently being performed and the results will be included in the next report.

KEY RESEARCH ACCOMPLISHMENTS

Aim 1

a. Immunohistochemistry for p63 was performed in the urogenital sinus of 10 wild-type male embryos at 18dpc. Our results demonstrated that during this early stage of prostate development, all the cells in the buds are p63 positive.

b. We are still working on the optimization of the *in situ* hybridization protocol for the detection of p63 transcripts in skin and prostate tissue sections.

Aim 2

a. We generated 56 chimeric mice by injecting the selected subclone of ROSA 26 ES cells in blastocysts generated by crossing p63 +/- females with p63+/- males.

b. The 56 chimeric mice were analyzed at 18.5 dpc by X-gal histochemistry and p63 immunohistochemistry.

c. We demonstrated that p63 function is cell autonomous.

d. We demonstrated that skin, limb, and prostate defects in p63-/- mice can be both partially and completely rescued (8 p63-/- rescued chimeras identified).

e. We demonstrated that prostate buds are exclusively populated by ROSA26 cells. This result establishes that p63 is required for the development of all the prostate epithelial cells at this early stage of development.

REPORTABLE OUTCOMES

Manuscripts sponsored by the DADM17-01-1-0051 proposal:

1. Lindeman N, Waltregny D, **Signoretti S**, Loda M. Gene transcript quantitation by real time RT-PCR in cells selected by immunohistochemistry-laser capture microdissection. Diagn Mol Pathol. 2002;4:187-92.

2. Weinstein MH, **Signoretti S**, Loda M. Diagnostic utility of immunohistochemical staining for p63, a sensitive marker of prostatic basal cells. Mod Pathol 2002;12:1302-1308.

3. **Signoretti S**, di Marcotullio L, Richardson A, Ramaswamy S, Carrano A C, Isaac B, Rue M, Monti F, Loda M and Pagano M. Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer. J Clin Invest. 2002;110:633-41.

4. Garraway LA[#], Lin D[#], **Signoretti S**, Waltregny D, Dilks J, Bhattacharya N, Loda M. Intermediate Basal Cells of the Prostate: *In Vitro* and *In Vivo* Characterization. Prostate. 2003;15;:206-18.

5. Graner E, Tang D, Rossi S, Weinstein LJ, Lechpammer M, Huesken D, Zimmermann J, **Signoretti S**, Loda M. The isopeptidase USP2a regulates the stability of Fatty Acid Synthase in prostate cancer. Manuscript submitted.

Abstracts sponsored by the DADM17-01-1-0051 proposal:

1. 9th Prouts Neck Prostate Cancer Meeting. Invited speaker: The basal cell marker p63 and prostate stem cells.

CONCLUSIONS

The major achievement is the demonstration that p63 function is cell autonomous and that skin, limb, and prostate defects in p63-/- mice can be rescued by injecting ROSA26 ES cells into 3.5 dpc blastocysts. Eight rescued p63-/- chimeric embryos at 18.5 dpc were identified by X-gal histochemistry and p63 immunohistochemisty studies. Analysis of the UGS epithelium and prostate buds of the rescued chimeras demonstrated that p63 is required for the development of all the epithelial cells that constitutes the prostate at this early stage of development. However, since we have also shown that all the cells that populate the prostate buds at this stage have a basal cell phenotype (and do express p63), no information regarding secretory cells can be obtained by analyzing the rescued mice at this embryonic stage. In order to overcome this problem, we have performed a second experiment in which the chimeric mice will be analyzed at 7 weeks of age. The analysis is currently being performed and the results will be included in the next report.

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